BIOCHEMICAL OXIDATION OF DAIRY WASTES. I. METHODS OF STUDY

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Microbial oxidation of dairy waste is being investigated as a possible rapid aeration process for the disposal of such wastes. The composition of dairy wastes varies in total solids from less than 1 per cent to more than 4 per cent of that of milk (1), depending on waste-saving practices advocated by Trebler and Harding (11). The total solids in milk have been reported as 125,000 p.p.m. by Eldridge (2) and 131,500 p.p.m. by Bloodgood (1); hence, a waste containing 1 per cent milk would have about 1,300 p.p.m. total solids, almost all of which are organic solids. The constituents of whole milk vary within wide limits (1) (4); Table I gives the approximate average composition (11). The fat is rapidly coagulated during aeration, and the soluble constituents left are mainly protein and lactose. It was decided, therefore, that a solution of dried skim milk would be suitable for preliminary surveys of the activities of various microorganisms. Table I includes the average composition of dried skim milk and a synthetic dairy waste prepared by dissolving 1 gm. of this product in 1 l. of water. It will be noted that the fat-free organic solids in the milk waste and in the skim milk preparation are 830 p.p.m. for the former and 874 p.p.m. for the latter.

A waste of this type has an average 5-day B.O.D. about 3 or 4 times as great as that of domestic waste. According to Galligan and Levine (5), the treatment of dairy wastes in a disposal plant designed for municipal sewage may cause severe operating difficulties. Moreover, many plants are in communities without facilities for treatment. The high oxygen demand and ready biochemical availability of the protein and carbohydrate of milk indicate the desirability of developing an aerobic treatment specifically designed for milk wastes. In this investigation, the ability of various organisms to alter the B.O.D. under aerobic conditions was followed by determining changes in individual constituents of the waste. Thus, the utilization of the organic constituents for synthesis of cell substance or their partial or complete oxidation could be

TABLE I.—Average Composition of Milk and Dried Skim Milk

Constituent	Whole Milk (%)	Dried Skim Milk (%)	Solution Containing	
			1% Milk (p.p.m.)	0.1% Dried Skim Milk (p.p.m.)
Fat	3.9	0.9	390	9
Protein	3.2	36.9	320	369
Lactose	5.1	50.5	510	505
Ash	0.7	8.1	70	81
Total Solids	12.9	96.4	1,290	964
Organic Solids	12.2	88.3	1,220	883

followed. The changes in oxygen demand due to lactose, protein, and nonprotein nitrogenous substances could be followed, as well as the total oxygen demand and pH. The methods used and their application to the problem are presented.

Methods

Chemical Oxidation Demand (C.O.D.)

A rapid chemical method developed by Eldridge and associates (3) and hitherto unpublished has been applied in this study. They conducted B.O.D. tests on fresh and fermented waste prepared from skim milk, with the results shown in Figure 1. On fresh wastes, the C.O.D. was essentially the same as the 20-day B.O.D. The rate curve of the fermented waste differed from that of the fresh waste. The C.O.D. appeared to be comparable to the point on the curve where second-stage oxidation had taken place. Eldridge has obtained additional data showing that with milk wastes, the suggested C.O.D. test appeared to give more consistent and reliable results than the 5-day B.O.D. test. Table II gives results obtained in the laboratory on freshly prepared solutions. The C.O.D. shows good agreement with the 20-day B.O.D. for the milk products tried, but no comparison exists between the 5-day B.O.D. and 68 per cent of the C.O.D. The need of a rapid chemical test that gives reproducible results comparable

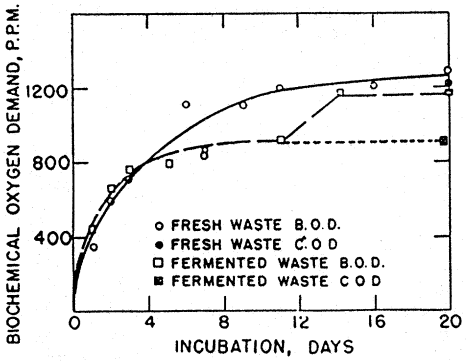

FIGURE 1.—B.O.D. rate curves of fresh and fermented skim milk (see text).

TABLE II.—Comparison of Oxygen Demands of Solutions Determined Chemically and Biologically

Type of Solution	C.O.D. (p.p.m.)		B.O.D. (p.p.m.)	
	Total	68%	20-day	5-day
Skim Milk	1,052	715	1,056	636
Lactose	516	351	519	431
Casein	604	412	639	327

to those of the B.O.D. test is generally recognized, and methods have been reviewed by Rhame (8) and Ingols and Murray (6). The fairly homogeneous character of dairy wastes permits the use of a chemical method, with satisfactory results. Therefore, the data herein are reported as the chemical oxygen demand, which may be considered for general purposes as the 20-day B.O.D. Details of Eldridge's chemical oxidation method, which is a modification of Rhame's chromic acid method, are as follows:

Reagents: 1. Dichromate oxidizing agent is prepared by dissolving 2.5 gm. of potassium dichromate in a mixture of 500 ml. each of concentrated sulfuric acid and 85 per cent orthophosphoric acid. Triturating the dichromate with small quantities of the orthophosphoric acid aids solution. The oxidizing agent is filtered through glass wool before it is used.

2. Potassium iodide solution is made by dissolving 55.3 gm. in 200 ml. of water.

3. Sodium thiosulfate, 0.05 normal, is prepared by dissolving exactly 12.41 gm. in water and making up to 1 liter.

4. Soluble starch solution.

Procedure: 1. Place exactly 50 ml. of the dicromate oxidizing solution in a 500-ml. Phillips beaker.

2. Transfer 5 ml. of the waste sample to the beaker.

3. Place the beaker on a hot plate and suspend a thermometer in the flask so that it does not touch the bottom.

4. Heat uniformly; a temperature of 165° to 170° C. should be reached in 5 to 6 min.

5. Immediately remove the beaker from the heat, remove the thermometer and cool the flask. Cooling to room temperature should take about 5 min.

6. Add 200 ml. of distilled water to the beaker and again cool to room temperature in a water bath.

7. Add 10 ml. of potassium iodide solution and titrate immediately with the 0.05 N thiosulfate solution, adding starch near the end point. The color change is from dark blue to pale blue-green.

8. A blank determination using 5 ml. water is run at the same time. All determinations are made in duplicate.

9. Total oxygen demand, in p.p.m., is equal to 80 times the difference in ml. used by the water blank and sample. The calculations are as follows:

$$\frac{D \times 0.05 \times 8}{5} \times 1{,}000 = D \times 80$$

In which D is the difference in reading between the blank and the sample; 0.05 is the normality of the thiosulfate used; 8 is the milli-equivalent of oxygen; 5 is the volume of sample taken; and 1,000 is the conversion to one liter, giving milligram per liter or p.p.m.

Lactose

The procedure for determining the oxygen demand due to lactose is essentially the micromethod of Stiles, Peterson, and Fred (10) adapted to this study as follows: Place exactly 15 ml. of waste solution in a 25-ml. volumetric flask and neutralize to phenolphthalein. Add 1 ml. of solution containing 33 per cent basic lead acetate and shake. Remove excess lead by adding 3 ml. of solution containing 10 per cent disodium phosphate, mix thoroughly, and make up to volume. Allow coagulum to settle. Pipette 5 ml. of clear supernatant liquid into a 50-ml. test tube, add 5 ml. microreagent,* stopper with loose-fitting cork, and heat in a boiling water bath for exactly 15 min. Cool rapidly, add 5 ml. N sulfuric acid, shake, and after standing for 1 min. titrate with 0.005 N sodium thiosulfate, using starch as an indicator. A similar determination is made on a water blank. The difference in the two readings is an indication of the amount of reducing sugar present, and the lactose value in p.p.m. is obtained from a graph prepared by using known amounts of lactose hydrate. The major ingredient of the unfermented synthetic waste used in these studies is lactose, amounting to 50 per cent of the total solids. Therefore, in a solution containing 1 gm. of skim milk per liter, 500 p.p.m. lactose hydrate is present. The complete oxidation of this lactose would require 561 mg. of oxygen, or 561 p.p.m. This gives a theoretical factor of 1.123 for obtaining the oxygen demand of lactose, the same as that obtained by the chemical oxidation test. Thus, that part of the oxygen demand due to the sugar can be calculated after the amount of sugar present in the waste is determined.

Protein

The procedure for determining the oxygen demand due to protein is based on that of Robinson and Hogden (9), who determined the protein content of blood serum. The intensity of the biuret color is measured at a wave length of 560 millimicrons, and the protein content is obtained by comparison with a reference graph.

Place 40 ml. of the sample in a centrifuge tube, add 2 ml. of trichloro-

* Microreagent: Dissolve 40 gm. anhydrous sodium carbonate in 400 ml. warm water. Now dissolve 5 gm. copper sulfate and 7.5 gm. tartaric acid in about 150 ml. water and add to the sodium carbonate solution with stirring. In 250 ml. water, dissolve 10 gm. potassium iodide, 0.7 gm. potassium iodate, and 18.4 gm. potassium oxalate; then add the mixture to the alkaline copper solution, cool, and dilute to 1 liter.

acetic acid solution containing 2 gm. of acid, mix, and place in a water bath at 60° to 70° C. for 5 to 10 min. Cool in water bath and centrifuge for 15 min. at 2,500 r.p.m. (In the equipment used, this was a relative centrifugal force of 1,700.) Pour off the supernatant liquor. Add 5 ml. of 3 per cent sodium hydroxide to the coagulum and dissolve the protein, using gentle heat if necessary. Add 35 ml. of sodium hydroxide solution; add 1 ml. of 20 per cent copper sulfate solution, stopper, and shake vigorously for 1 min. Let stand for 1 hr.; centrifuge about 10 min. to pack the precipitate, then filter the supernatant liquid through a sintered glass filter of medium porosity, collecting the clear filtrate in a dry tube. Using a 60-mm. cell, determine the density of color spectrophotometrically at a wave length of 560 millimicrons. Convert the reading to protein value by means of a reference graph (Figure 2), in which the density values are related to mg. of protein per liter (p.p.m.) when using a 40-ml. sample.

In establishing the reference graph, 1 gm. of dried skim milk was dissolved in 500 ml. of water. The proteins were precipitated in a 5 per cent concentration of trichloroacetic acid, centrifuged, and collected on a sintered-glass filter. The proteins were dissolved with 3 per cent sodium hydroxide and made up to a liter volume with the alkali solution. Different amounts of this stock solution were transferred to large centrifuge tubes and made up to 40-ml. volumes with 3 per cent sodium hydroxide. The blue color was developed and filtered as above, and the density determined on the spectrophotometer. The nitrogen contents of these blue biuret solutions were determined by microkjeldahl method, and the protein was estimated by the factor 6.25. The values were plotted as shown in Figure 2. The amount of oxygen required for the oxidation of protein was determined by the proposed C.O.D. method on solutions containing known amounts of commercial casein, protein coagulated from casein, and protein coagulated from skim milk. The results ranged from 1.41 to 1.44; the average was 1.42. In the synthetic waste solution containing 356 p.p.m. protein, the oxygen demand was calculated to be 505 p.p.m.

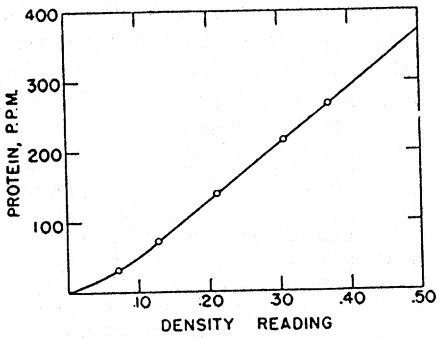

FIGURE 2.—Relationship between milk protein and biuret density values, as measured spectrophotometrically at 560 millimicron; volume of waste, 40 ml.

Nonprotein Nitrogenous Compounds

Protein determinations made directly on the fresh waste without first coagulating the protein gave values much higher than the protein in the waste. As lactose was the other major ingredient in the waste, a study was made to determine its effect on the protein reading. To different amounts of casein solution, increments of lactose solution were added. Sodium hydroxide was added to give a concentration of 3 per cent in the final 40-ml. volume. The blue color was developed, and the apparent protein values were obtained from Figure 2. Increased values were obtained with increments of lactose in each concentration of protein. The corrections applied when lactose was present are given in Figure 3. Thus, if 125 p.p.m. lactose was present, 70 p.p.m. was deducted to correct for the sugar. An estimation of the

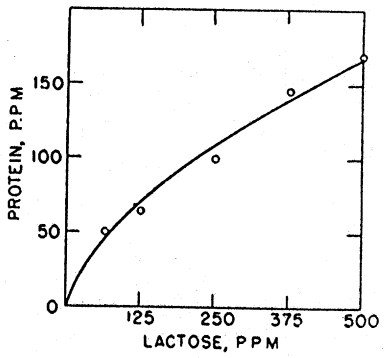

FIGURE 3.—Correction values for biuret-protein values when lactose is present in the solution.

nonprotein nitrogenous substance was obtained in a 20-ml. sample to which 20 ml. of 6 per cent sodium hydroxide were added, and the blue color developed. The correction for lactose was made, and then the protein found by coagulation was subtracted, leaving an approximate value for the nonprotein nitrogenous fraction.

This fraction may be considered essentially as including products of proteolysis, such as peptides, which give a color with the biuret reaction and are closely related to proteins. Therefore, the same conversion factor as for protein, 1.42, has been used to calculate the oxygen demand of this fraction.

Other Substances

The sum of the oxygen demands of the lactose, protein, and nonprotein nitrogenous compounds is usually less than the total oxygen demand. In fresh synthetic waste, the difference is small, but as the material is acted on by the microorganisms the difference becomes greater. This fraction includes the oxygen required to oxidize nonnitrogenous cell substances synthesized by the microorganisms; products of lactose breakdown, such as acids; and free amino acids that do not give a color in the biuret reaction, as well as other miscellaneous products of the organisms.

Experimental Results

Several 400-ml. portions of a solution containing 1 gm. dried skim milk per liter were distributed in a number of 500-ml. gas washing bottles having fritted-glass discs. After steam sterilization, each bottle was inoculated with 20 ml. of a desired culture grown for about 65 hr. in a shallow layer of a similar skim milk solution. These bottles were kept at room temperature and aerated at the rate of 400 ml. of filtered air per minute. Silicone antifoam was added, and the exit tube of each aerator was connected to a sterile flask to collect any escaping foam. At 24-hr. intervals, a complete unit was removed for analyses. The solution was made up to volume, and passed through glass wool, and the necessary amounts were removed for sugar and acid determinations. The insoluble material adhering to the glass wool and to the aerator was dissolved with sodium hydroxide and, after the excess caustic was neutralized with sulfuric acid, was added to the remainder of the solution. The protein analyses and C.O.D. tests were made on this solution. Three series of flasks were

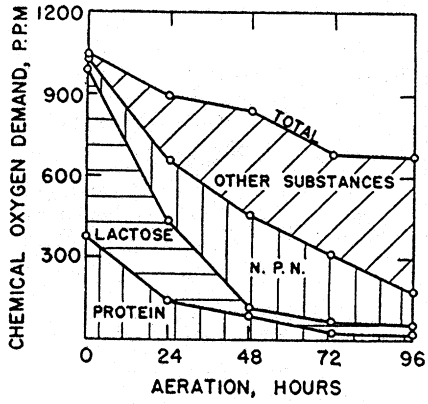

FIGURE 4.—Changes in the proportion and in the composition of dairy waste caused by the aerobic action of *B. polymyxa*. Areas between the curves represent the chemical oxygen demand of the ingredients; that of the whole waste is represented by the total area.

prepared and inoculated with *Bacillus polymyxa, Saccharomyces fragilis,* and a small rod isolated from cow manure. The action of each organism on the constitutents of the waste as measured by the oxygen demand over 96 hr. of aeration has been plotted.

B. polymyxa (Figure 4) was selected because of its great proteolytic activity. This action was confirmed on this waste, in which only about 15 per cent of the proteins still remained after 48 hr. Disappearance of lactose was also rapid. The pH at this time was 5.2, but reached 5.8 at 96 hr. The total oxygen demand, however, had decreased only about 21 per cent in 48 hr. and 37 per cent in 96 hr. Although the original ingredients of the milk had disappeared to a great extent, the action was primarily hydrolytic, as significant portions were found in the nonprotein nitrogenous fraction and in the fraction consisting of acids and other metabolic products.

S. fragilis acted somewhat differently, as seen in Figure 5. The protein fraction remained fairly constant, being converted to cell substance, as evidenced by the vigorous growth.

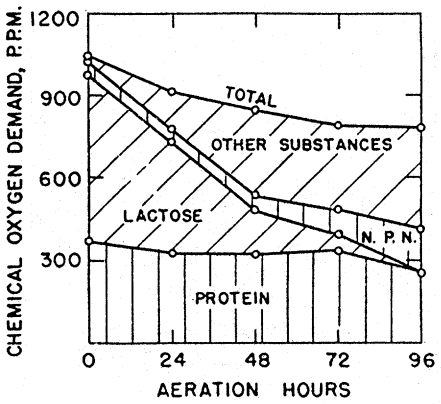

FIGURE 5.—Changes in the proportion and in the composition of dairy waste caused by the aerobic action of the yeast, *S. fragilis*. Areas between the curves represent the chemical oxygen demand of the ingredients; that of the whole waste is represented by the total area.

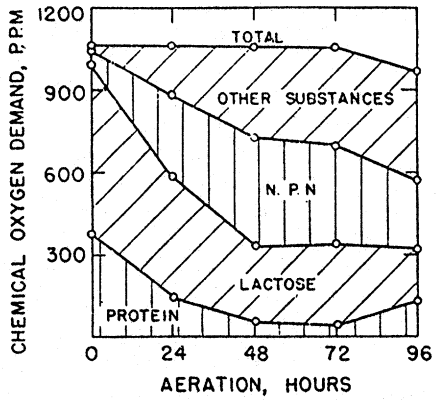

FIGURE 6.—Changes in the proportion and in the composition of dairy waste caused by the aerobic action of a coli organism. Areas between the curves represent the chemical oxygen demand of the ingredients; that of the whole waste is represented by the total area.

Lactose was utilized fairly readily, and the nonprotein nitrogenous fraction remained small. Other substances were produced, and the pH decreased from 5.7 to 5.3. The total oxygen demand had been decreased 26 per cent in this experiment. If the protein were considered as one-half the yeast cell (7) and mechanical means of removing the yeast were used, the expected reduction in oxygen demand would be about 70 per cent. In other experiments, tests made on the supernatant liquid after centrifuging showed a reduction of about 75 per cent in C.O.D.

The third series of aerators were inoculated with a small rod isolated from cow manure, tentatively identified as of the coli group. As shown in Figure 6, there was little change in the total oxygen demand, although the protein and lactose were attacked. At 48 hr., most of the protein had disappeared, but the nonprotein nitrogenous fraction had increased; more than 50 per cent of the lactose had been converted to other substances. At 96 hr., there occurred a slight decrease in oxygen demand, accompanied by an increase in protein.

Discussion

In a search for organisms and conditions that will decrease the oxygen demand of diluted dairy waste, it is desirable to determine rapidly the constituents of the waste and the oxygen demand of these constituents in addition to the total oxygen demand. The chemical methods used in these studies are fairly rapid and reproducible, and therefore can be used to follow a rapid aeration process. They can determine changes in individual ingredients and their utilization for synthesis of cell substances, or their complete or partial oxidation.

The pollution effect of the dairy waste may be obtained by the C.O.D. test, but the proportional amount contributed by the ingredients of the waste must be determined separately. A decrease in lactose may cause a decrease in total oxygen demand if the sugar is oxidized, or little change may occur if it is converted to other products of similar oxygen demand or if it is used for protein synthesis. Ideally, lactose or its derivatives should be completely oxidized to carbon dioxide and water. In the same way, the protein of the milk may be oxidized to different degrees, and the nitrogen used to synthesize cell protein. It may be hydrolyzed without altering its effect on the total oxygen demand of the waste.

The ideal condition would exist in milk waste with its soluble constituents if the C.O.D. of the waste and its ingredients would converge to zero in a relatively few hours. This can only be approached, as residual cell substance itself has an oxygen demand. Therefore, it becomes necessary to establish conditions that will oxidize much of the waste solubles as completely as possible without the production of other soluble products such as lactic acid, amino acids, etc. In addition, the soluble products not oxidized must be converted to an insoluble form, such as cell substance, which can be readily removed from the solution, leaving an effluent of low oxygen demand. The methods proposed offer a means for following these changes.

Summary

Methods are given for following the changes in oxygen demand of the major ingredients of dairy waste—protein and lactose—during rapid aeration.

A rapid chemical oxygen demand (C.O.D.) test giving consistent results with dairy wastes is described in detail.

Applications of these methods to the biochemical oxidation of 0.1 per cent skim milk by three organisms for 96 hr. are presented.

The organisms used were *Bacillus polymyxa*, *Saccharomyces fragilis*, and a coli organism.

Acknowledgments

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